

MicroRNA expression differs in cutaneous squamous cell carcinomas and healthy skin of immunocompetent individuals

Subtitle: Expression of miRNA in cutaneous SCC

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Abstract

Cutaneous squamous cell carcinoma (cSCC) is one of the most common skin cancers, but the influence of microRNA (miRNA) expression has only been sporadically analysed. We hypothesized that miRNAs are differentially expressed in cSCC and hence influence its development. We therefore isolated total miRNA from well-differentiated cSCCs and from controls without SCC.

Expression analyses of 12 miRNAs showed three significantly differentially expressed miRNAs. We identified a significant upregulation of the miR-21 and the miR-31, a proto-oncogene like miR-21. While the upregulated expression of miR-21 has been known for some time, the increased expression of miR-31 was never shown so clearly. Furthermore we showed the upregulation of miRNA-205, which has never been described before. The miR-205 induces specific keratinocyte migration and could be a characteristic marker for cSCC. It has to be determined in following studies whether these upregulated expressions are specific for cSCC and if so, for which cSCC-stages.

Cutaneous squamous cell carcinoma (cSCC) is one of the most common skin cancers and leading to death in 0.5 to 2% of the cases in Europe (1-3). Numerous pathways are reported to be involved in its development, but the influence of microRNAs (miRNA) has only been sporadically analysed (4-7) and the relation between their expression and cSCC development has only scarcely been looked at (8, 9).

We hypothesized that miRNAs are differentially expressed in cSCC and hence influence its development. We therefore isolated total RNA of 10 formalin-fixed and paraffin-embedded (FFPE) well-differentiated cSCCs from 9 immunocompetent individuals without inherited cancer predisposition. Additionally, a HPV analysis was performed (suppl. material). As a control, 11 total RNA samples were isolated from healthy epidermis from patients without SCC. The selected 12 miRNAs (Table S1) were previously reported to be differentially expressed in several types of cancer or are involved in epidermis differentiation (10-12). Normalization of miRNA expression was carried out against four different snoRNAs (U18, U24, U44, and U48) reported to be invariably expressed in skin (13). The study was approved by the local Ethics Committee of Basel and performed according to the declaration of Helsinki.

Expression analyses showed three significantly differentially expressed miRNAs (miR-21, miR-31, miR-205), which were all upregulated in cSCCs (Fig. S1). The normalization showed significant results for miR-21 and -31 against all four snoRNAs whereas data for miR-205 was significant only relative to three snoRNAs (Fig. S2). Three miRNAs (miR-17-5p, 106a, -155) were significantly increased only when normalized against some snoRNAs (Fig. S3). A significant decrease was found in let-7b-5p when normalized against U44. Two miRNAs (miR-184 and -206) were detectable neither in the control skin nor in the cSCCs within a reliable cycle number of less than 40 cycles. HPV-DNA was not detectable in any cSCC sample.

The oncogenic potential of miR-21 is well known and reported in many other cancers (14). The high induction of miR-21 in the well-differentiated cSCCs vs. normal skin confirms previously published data (8). Concurrent with our examinations an upregulation of miR-31 in cSCCs was reported (Table 1) (9). Like miR-21, miR-31 is a proto-oncogene (15), but its effect seems to be versatile in several cancers and cancer stages (16-20). In cSCC the function of miR-31 has yet to be clarified.

In contrast to previously published data on cSCCs the expression of miR-205 is increased in our samples when normalized to three snoRNAs (U18, U24, and U48) (Table 1, Fig S1) (8, 9). These different results may also be influenced by the method and normalization. Increased levels of miR-205 were shown in aggressive oral SCCs (21). Supporting a proto-oncogene function, miR-205 enhances the migration of keratinocytes in the cutaneous epidermis (22). Previous findings revealed that miR-205 is antagonized by miR-184 (21) which is in accordance with our results of an undetectable miRNA-184 level. Previous reports on the expression of miR-184 diverge (8, 9).

Our analyses showed no difference in the expression of miR-203 in cSCC and in normal skin, as also reported by Xu *et al.* 2012. In contrast, Dziunycz *et al.* 2010 reported this same miRNA to be downregulated in cSCC. The tumour-suppressor miR-203, a skin and keratinocytes-specific miRNA, mediates *p63* degradation (11, 23-25). This is in agreement with a decreasing expression of miR-203 with lower differentiation stage of the tumour (8). As we aimed for examination of early and clearly well-differentiated cSCC and found no regulation of miR-203 expression, the miR-203 level could be an indicator for the differentiation grade of the tumour. Consistently, the expression of miR-34a, another tumour-suppressor downstream of *p63*, is not differentially regulated in our cSCC tissues.

As reported previously and confirmed by our results, the choice of normalization genes has a strong impact on the results. The results show a clear involvement of several unsuspected miRNAs in cSCC development. As it is known for other cancers, we assume that the miR-levels change during carcinogenesis. This hypothesis could be tested by analysing actinic keratoses and all stages of cSCC. An expression signature of miRNA in different cSCCs could sharpen the individual prognosis of the metastatic risk. Finally, deeper understanding of differential miRNA expression in cSCC development may lead to the development of new and better targeted therapies (19). HPV is supposedly a risk factor for cSCC development. However, only half of cSCCs reveal HPV-DNA (6, 26, 27) whereas no HPV-activity could be detected (28). Evidences show that HPV effects are boosted during actinic keratosis (29).

In conclusion, our results reveal new miRNA players in the carcinogenesis of cSCCs. Concurrent with Xu *et al.* we identified a significant upregulation of the miR-31 in well differentiated cSCCs. Furthermore we showed the differential upregulation of miRNA-205. Whether miR-205 has an influence on the development of cSCCs

must be clarified in subsequent studies. Significant increase of miR-21 in cSCC tissues could be confirmed by our observations. The question on which miRNAs are characteristically differentially expressed during cSCC development remains. For example, miR-21 is generally upregulated in proliferating tissues and miR-125a is downregulated in cutaneous non-tumorous proliferating tissues (30). In contrast, miR-205 induces keratinocyte migration (22) hence its expression level could be used as a specific marker for cSCCs development. Whether miR-31 upregulation is specific for cSCCs, for other types of SCCs or for other cancers has yet to be determined in additional studies. Data of all stages from precancerous lesion to metastasize cSCC along with the clinical phenotype are essential to achieve a better understanding of the carcinogenesis and aggressiveness of cSCC.

Our experiments reveal that some miRNAs, whose expression is described to be different in cSCCs by several authors, are possible key players in this cancer type and detectable by various methods. Uniform conditions could help to assess the relevance of miRNAs whose expression is differentially described. However, the clarification of the specific miRNA expression in different cSCCs could provide key information to understand the evolution of cSCCs and will ultimately enable us to develop novel potential for treatment of these cancers.

Author contributions:

Christelle Bruegger performed research and prepared the manuscript; Iris Spoerri performed research; Werner Kempf did the HPV analyses; Andreas Arnold surveyed the cSCCs; Peter Itin designed the study and contributed to data analysis; Bettina Burger designed the study, analysed the data and contributed to the manuscript preparation.

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Table 1. Summary of the analysed miRNAs and results of their regulation compared to the findings of other groups. Different findings are highlighted in bold letters.

<i>miRNA</i>	<i>Dziunycz et al. 2010</i>	<i>Xu et al. 2012</i>	<i>presented study</i>
let-7a-5p	not analysed	slight downregulation^b	no different regulation
let-7b-5p	not analysed	slight downregulation^b	no different regulation
miR17-5p	not analysed	no different regulation	no different regulation
miR21-5p	upregulation	upregulation	upregulation
miR31-5p	not analysed	upregulation	upregulation
miR34a-5p	not analysed	no different regulation	no different regulation
miR106a-5p	not analysed	not analysed	no different regulation
miR155-5p	not analysed	no different regulation	no different regulation
miR184	upregulation^a	no different regulation^b	not detectable
miR203	downregulation^a	no different regulation	no different regulation
miR205-5p	no different regulation^a	no different regulation^b	upregulation
miR206	not analysed	no different regulation ^b	not detectable

^a It is not mentioned whether only tumour-specific lymphocyte-reduced / -free parts of the tumour or the complete tumour are isolated. Normalization was only performed to U18.

^b Xu et al. investigated the complete tumour material by the multiplex RT TaqMan MicroRNA Low Density Array (TLDA) (Applied Biosystems)

Supplementary Material

Experimental Design. Cutaneous SCC samples were selected from 9 anonymous immunocompetent individuals without inherited cancer predisposition from the Dermatology Department of Basel. Total RNA from cSCC as well as from anonymous healthy controls was isolated using the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Ambion) according to the manufacturers' instructions. The quantification of 12 specific miRNAs was performed in triplicates by TaqMan® MicroRNA assays (LifeTechnologies) according to the manufacturer's protocol.

Investigation of all cSCCs for the presence of human papillomavirus (HPV) was done by polymerase chain reaction as described before (1).

Statistical analysis. Relative miRNA expression was determined using the $2^{-\Delta\Delta C_T}$ -method (2). Each examined miRNA was normalized against every analysed snoRNA. Fold induction was obtained for significantly expressed miRNAs by the difference of the normalized mean expression between cSCCs and controls. To exclude possible artifacts due to the regulation of the normalization genes we confirmed the results by using qbasePLUS® (Biogazelle), which applies the mean expression value for normalization (3). It also performs the necessary statistical analyses e.g. the unpaired t-test and multiple testing correction. The reference target stability was proven by qbase Plus®. The snoRNAs U24 and U48 emerged to be the most stable reference genes in our study ($M < 0.7$) whereas the U18 and U44 were less stable ($M < 1.0$). This finding correlates with the different results which we obtained for the normalization of several miRNA (see Fig. S3).

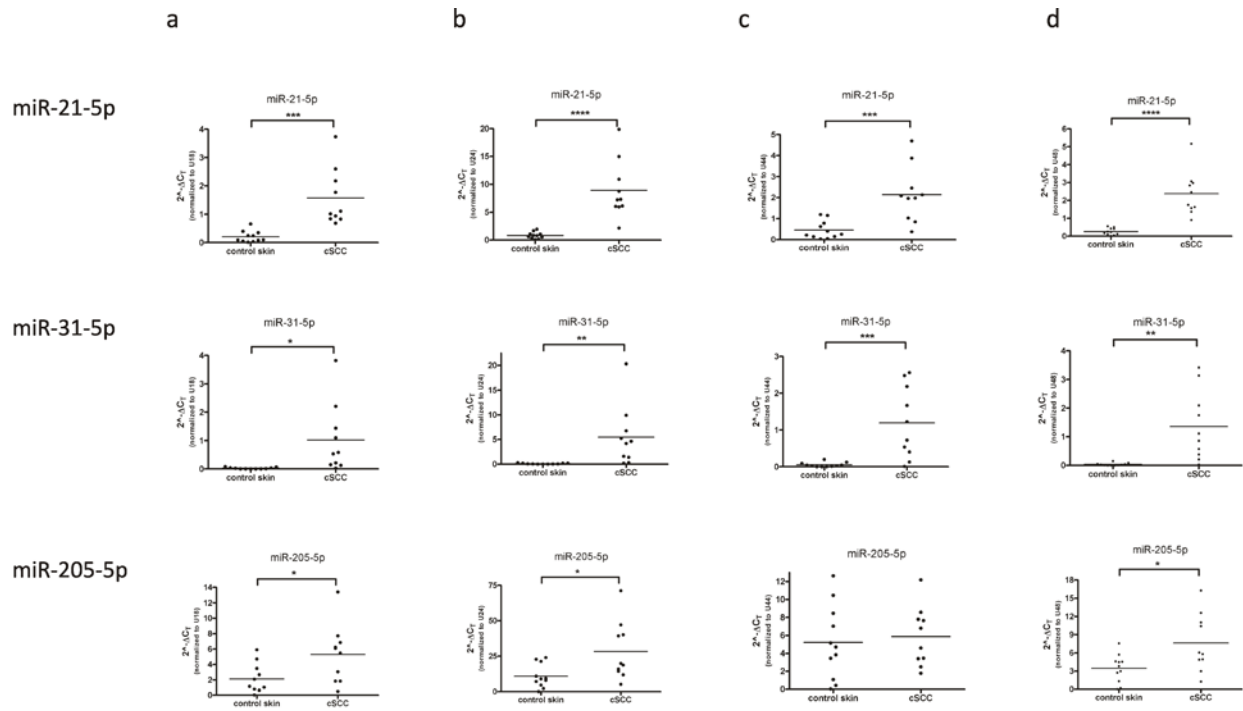


Fig. S1. Level of miR-21-5p, miR-31-5p, and miR-205-5p in control skin and in cSCC was determined by qPCR and normalized to U18 (a), U24 (b), U44 (c), and U48 (d) resulting in variable significances. The mean is indicated by the horizontal line. *P<0.05, **p<0.01, ***p<0.0001

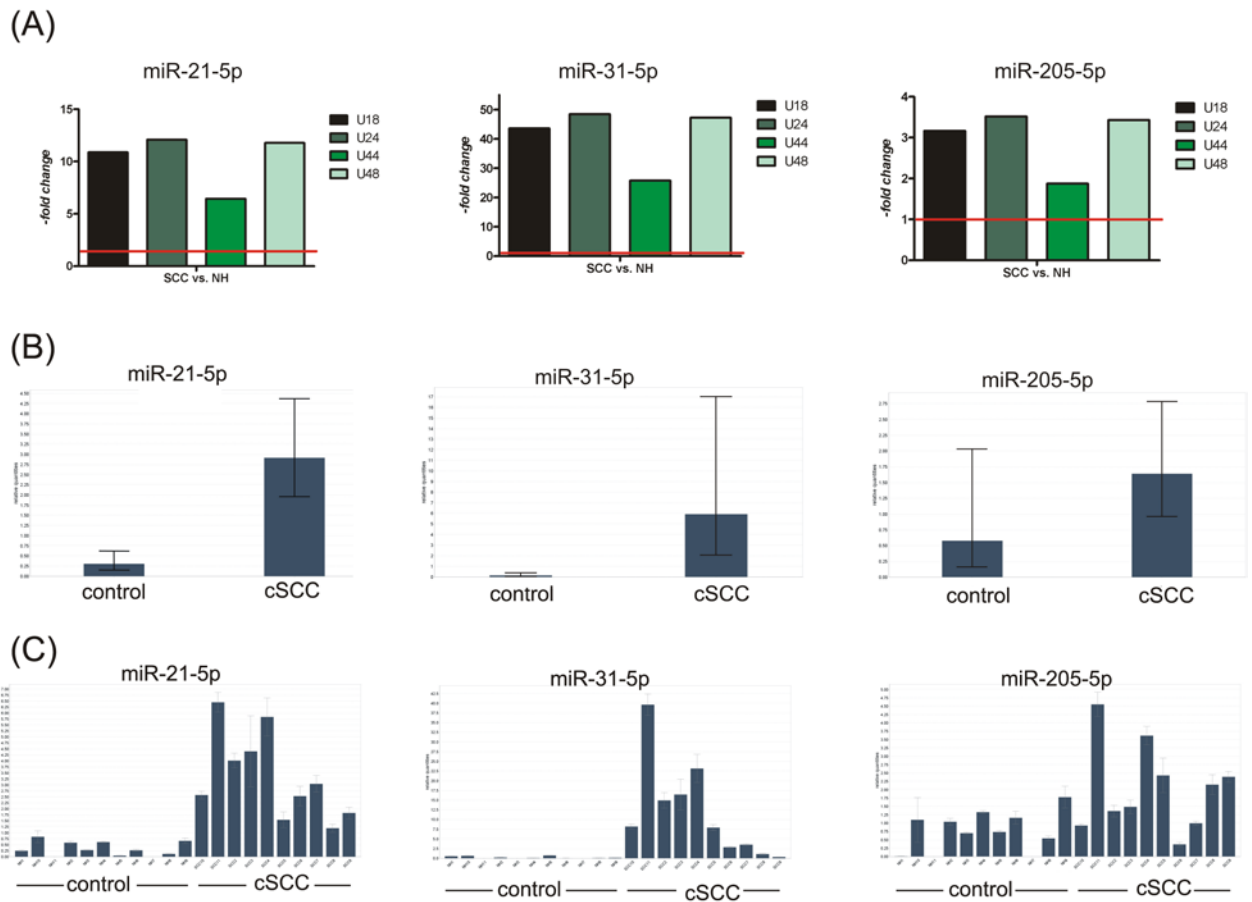
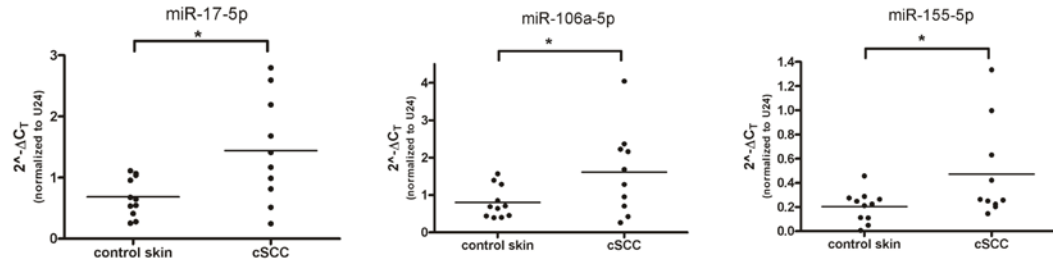
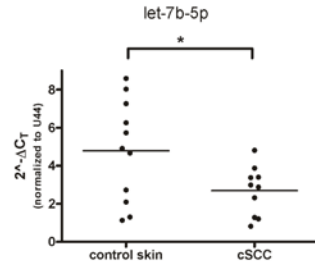


Fig. S2. (A) N-fold induction of miR-21-5p, miR-31-5p, and miR205-5p in cSCC vs. control skin. Difference of specific miRNA expression between cSCC and control skin was determined for each normalization resulting in variable data. Each miRNA was normalized to all snoRNAs resulting in variable significances. The red line marks the value 1. (B, C) Data analysis was additionally performed by qbase PLUS (BioGazelle), which identified the U44 to be not valuable for normalization. Results also showed significant upregulation for the three miRNAs, whereby the high SD points to the variability of the cSCCs.

normalized
to U24



normalized
to U44



normalized
to U48

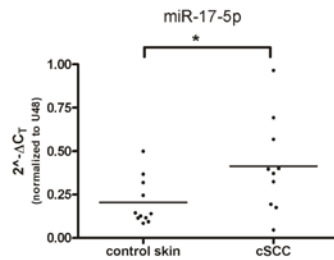


Fig. S3. Expression levels of four miRNAs, which show a significant increase (miR-17-5p, 106a-5p, and 155-5p) or decrease (let-7b-5p) only when normalized against U24 and/or U48. The mean is shown by the horizontal line.

Table S1. Means of $2^{-\Delta C_T}$ of all investigated miRNAs normalized against four snoRNAs from cSCC and from control skin.

miRNA	normalization to	mean $2^{-\Delta C_T} \pm \text{SEM}$		p value (unpaired t-test)	-fold change
		cSCC	control skin		
let-7a-5p	U18	0.794 \pm 0.1735	1.094 \pm 0.2541	0.3503	0.77
	U24	4.443 \pm 0.9840	4.489 \pm 0.5382	0.9669	0.86
	U44	1.049 \pm 0.2344	2.226 \pm 0.5200	0.0608	0.46
	U48	1.280 \pm 0.2558	1.346 \pm 0.2171	0.8458	0.84
let-7b-5p	U18	2.145 \pm 0.4340	2.598 \pm 0.7448	0.6145	1.02
	U24	11.58 \pm 2.017	10.95 \pm 2.193	0.8376	1.13
	U44	2.698 \pm 0.4055	4.792 \pm 0.8039	0.0361	0.60
	U48	3.177 \pm 0.4418	3.252 \pm 0.5512	0.9179	1.10
miR17-5p	U18	0.2608 \pm 0.0482	0.1989 \pm 0.06639	0.4671	1.71
	U24	1.440 \pm 0.2738	0.6836 \pm 0.09524	0.0138	1.91
	U44	0.3123 \pm 0.0592	0.3357 \pm 0.08091	0.8205	1.01
	U48	0.4137 \pm 0.0854	0.2051 \pm 0.04091	0.0351	1.86

miR21-5p	U18	1.572 ±0.3152	0.2061 ±0.05996	0.0003	10.88
	U24	8.948 ±1.621	0.8490 ±0.1770	<0.0001	12.09
	U44	2.146 ±0.4178	0.4570 ±0.1282	0.0007	6.44
	U48	2.377 ±0.3892	0.2623 ±0.05677	<0.0001	11.79
miR31-5p	U18	1.022 ±0.3810	0.02534±0.008654	0.0128	43.57
	U24	5.458 ±1.916	0.09863 ±0.02964	0.0084	48.43
	U44	1.193 ±0.3079	0.05289 ±0.01891	0.0010	25.79
	U48	1.358±0.3812	0.03651 ±0.01389	0.0017	47.22
miR34a-5p	U18	0.0933 ±0.01846	0.0711 ±0.01776	0.3977	1.50
	U24	0.5742 ±0.1229	0.3037 ±0.05490	0.0518	1.67
	U44	0.1498 ±0.04625	0.1561 ±0.04227	0.9211	0.89
	U48	0.1432 ±0.04089	0.09516 ±0.02133	0.2981	1.45
miR106a-5p	U18	0.2910 ±0.06611	0.2528 ± 0.1075	0.7713	1.54
	U24	1.613 ± 0.3614	0.8046 ±0.1280	0.0413	1.72
	U44	0.3135 ±0.04994	0.4096 ±0.1231	0.4939	0.91
	U48	0.4238 ±0.07578	0.2432 ±0.05739	0.0674	1.67
miR155-5p	U18	0.07741 ±0.01736	0.05424 ±0.01922	0.3857	2.33
	U24	0.4723 ±0.1259	0.2041 ±0.03841	0.0472	2.59
	U44	0.1406 ±0.05500	0.1166 ±0.04345	0.7330	1.38
	U48	0.1392 ±0.04799	0.06527 ±0.01651	0.1333	2.52
miR184	U18	0.000237 ±0.00015	0.000170 ±0.0000836	0.6962	0.45
	U24	0.00155 ±0.00099	0.000693 ±0.000279	0.3970	0.50
	U44	0.000463 ±0.00030	0.000262±0.0000781	0.5085	0.26
	U48	0.000511 ±0.00046	0.000157 ±0.0000413	0.3732	0.48
miR203	U18	3.140 ±1.511	2.259 ±0.8971	0.6142	1.28
	U24	16.01 ±7.967	8.328 ±1.929	0.3393	1.42
	U44	2.934 ±0.8228	3.552 ±0.9020	0.6209	0.76
	U48	3.974 ±1.233	2.350 ±0.5339	0.2269	1.38
miR205-5p	U18	5.302 ±1.189	2.115 ±0.5690	0.0222	3.17
	U24	28.44 ±6.465	10.95 ±2.491	0.0170	3.52
	U44	5.846 ±1.036	5.212 ±1.237	0.6885	1.87
	U48	7.613 ±1.495	3.449 ±0.6949	0.0174	3.43
miR206	U18	0.000204 ±0.000068	0.000458 ±0.000127	0.1024	0.44
	U24	0.001294 ±0.000504	0.001959 ±0.000540	0.3815	0.49
	U44	0.000497 ±0.000275	0.000905 ±0.000297	0.3019	0.26
	U48	0.000382 ±0.000124	0.000536 ±0.000138	0.4209	0.48

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